云　南　植　物　研　究　2009，31 (5)：433～438
Acta Botanica Yunnanica　DOI：10.3724 SP.J.1143.2009.09096

稻属中一个高度保守和组成型表达酪氨酸 丝氨酸-酸酸双特性蛋白激酶基因的克隆与分析

杨明挚[1]，黄兴奇[2]，张汉波[1]，陈善娜[1]，杨红玉[3]，程在全[2]

(1 云南大学生命科学学院，云南 昆明　650091；2 云南省农业科学院种质资源与生物技术研究所，云南 昆明　650223；3 昆明学院生命科学与生物技术系，云南 昆明　650118)

摘要：从水稻中克隆了一个在稻属植物中高度保守和组成型表达的丝氨酸 苏氨酸蛋白激酶基因（OsSTK)。该基因包含两个外显子和一个114 bp 的小内含子序列，预测编码一个419个氨基酸的蛋白质。该基因推导的氨基酸序列与其它已知序列的一致性均低于 52%。利用从不同种和类型的野生稻克隆的部分该基因序列构建的系统树与野生稻的分类和进化关系相一致。OSPK N-端拥有一段富含丝氨酸、碱性氨基酸和带电荷氨基酸的特异性导肽序列，其中包含“GDGDGDGDG”短重复序列。由于该基因蛋白激酶结构域中的 VIb，VIII 和 XI 亚结构域中同时具有酪氨酸蛋白激酶和丝氨酸 苏氨酸蛋白激酶的特性，推测该基因可能同时具有催化酪氨酸和丝氨酸、苏氨酸磷酸化的双重功能。

关键词：稻属；丝氨酸 苏氨酸 蛋白激酶基因；保守性分析；转录分析

中图分类号：Q 943.2　　**文献标识码**：A　　**文章编号**：0253-2700 (2009) 05-433-06

Cloning and Analyses of a Dual Specific Serine Thronine Protein Kinase Gene with High Conservative and Constitutive Expression in *Oryza* (OsSTK)

YANG Ming-Zhi[1], HUANG Xing-Qi[2], ZHANG Han-Bo[1], CHEN Shan-Na[1],
YANG Hong-Yu[3], CHENG Zai-Quan[2]**

(1 *Life Science School*, *Yunnan University*, Kunming 650091, China; 2 *Biotechnology—Genetic Germplasm Institute*, *Yunnan Academy of Agricultural Sciences*, Kunming 650223, China;
3 *Biologydepartment*, *Kunming Institutey*, Kunming 650118, China)

Abstract: A cytoplasmic serine thronine protein kinase gene (OsSTK), had been cloned from *Oryza* genus. It was found high conservative and constitutive expression in *Oryza*. OsSTK gene had two exons, separated by 114 bp short intron. The open reading frame of OsSTK gene that predicted encoded a 419 amino acids protein. The amino acid sequence of OsSTK had low identities (less than 53%) with any other known protein kinase. The phylogenetic tree based on the partial DNA sequences of OsSTK from different species and types of wild rice and cultivated rice, was close to the taxation system of rice. Interestingly, OsSTK had a serine, including basic amino acids and charged amino acids abundant polypeptide with a “GDGDGDGDG” sequence at N-terminal that had not been found in any other genes. OsSTK may play dual specificity that phosphorylates both serine thronine and tyrosone, because the amino acids module of VIb, VIII and XI catalytic domain have both the serine thronine and tyrosine kinase characters.

Foundation items: National Natural Science Foundation of China (300660090), the Yunnan Natural Science Foundation of China (2006000007Q and 2008C004) and the department of science and technology of Yunnan province, China (2007PY01-24)

Author for correspondence; E-mail: czquan-99@163.com

Received date: 2009-05-15, Accepted date: 2009-06-19

作者简介：杨明挚 (1970-) 男，博士，副教授，从事植物生理与分子生物学研究。

Key words: *Oryza*; Serine thronine protein kinase gene (OsSTK); Conservation analysis; Transcription analysis

Protein kinase plays a central role in multiple biological processes, including growth, development, metabolism of cell, stress response and signal transduction (Natalia *et al.*, 1998). Protein kinase catalyze the reversible transfer of the γ-phosphate from ATP to amino acid side chains of proteins. Phosphorylation status of a protein has profound effects on its activity and interaction with other proteins. It is estimated that 1% to 3% of functional eukaryotic genes encode protein kinases. Enzymes belonging to the protein kinase superfamily are related by homologous protein kinase catalytic domains. Typically, eukaryotic protein kinases have been subdivided into those that phosphorylate Serine and or Thronine, those that phosphorylate Tyrosine and those that phosphorylate Histidine (Julie *et al.*, 1995). There are 12 conserved regions referred to as subdomains, some of which contain invariant or nearly invariant residues (Hanks *et al.*, 1991). Crystal structure determinations of protein kinases demonstrate the importance of these conserved residues in catalysis and conservation of overall three-dimensional structure (Julie *et al.*, 1995). Since the importance of protein kinase in plant biological process, the more we know about protein kinase, the more we will understand about the net work of cell metabolism and signal transduction. During the cloning of STK (serine thronine protein kinase) type resistance gene analogs from different species and types of wild rice, a highly conserved serine thronine protein kinase gene in *Oryza* has been identified. Here, we describe the cloning and characters of this novel protein kinase gene.

1 Materials and methods

1.1 Rice materials

Three wild rice species were collected from wild fields in Yunnan province, including *Oryza rufipogon* Griff, *O. officinalis* Wall ex watt and *O. meyerana* Baill. *O. longistaminata* was collected from Africa. *O. minuta* was from south Asia. Another type of *O. rufipogon* was from Jiangxi province, China. Four different types of *Oryza rufipogon* used in our research were: Yuanjiang type (collected from Yuanjiang in Yunnan province), red awn type (from Jinghong in Yunnan province), erect type (from Jinghong in Yunnan province), and Dongxiang type (from Jiangxi province). All of the collected cultivated rice varieties and wild rice species were grown in green house in Kunming of Yunnan province for further use. The young green leaves were used for genomic DNA and RNA extraction.

1.2 DNA isolation, finding and cloning of OsSTK gene

Plant genomic DNA was isolated by CTAB method from young plant leaves, according to the manufacturer's protocol (Doyle and Doyle, 1987). For the amplification of STK type resistance gene analogues, a pair of degenerated primers (KF and KR, table 1) was synthesized. 50 pmol L^{-1} of each primer (KF, KR) and 50 ng of genomic DNA were added to 50 μl of PCR reaction mixture with 5 μl of 10 × reaction buffer, 2.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} of each dNTP, and 4 units of Pfu DNA polymerase. Thirty cycles of PCR with denaturation at 95℃ for 40 s, reanealing at 55℃ for 40 s, and extension at 72℃ for 1 min, were performed in a DNA thermalcycler (PTC-200, MJ research). PCR products were purified and ligated to pGEM-T vector (Promega) and transformed into *E. coli* (DH5α), according to the manufacturer's protocol. Random selected plasmids containing inserts of the PCR products were sequenced by Sangon Ltd. China. Multiple DNA sequences analysis and alignment were made by the software DNASIS. Among the cloned STK type disease resistance analogues, we found one of them from *O. rufipogon*, *O. officinalis* and *O. meyerian*, respectively, shared highly identities. These DNA fragments may come from one homologous gene. By using one of these sequences as bait, searching in DNA database, we found a cDNA clone and a BAC clone, both has more than 99% identities with the bait sequence. According to the cDNA and BAC clone, we identified OsSTK gene.

1.3 Conservation analysis of OsSTK gene in *Oryza*

After the comparison to the OsSTK gene partial sequences from *O. rufipogon*, *O. officinalis* and *O. meyeriana*, a pair of specific primers (OTK1: 5′-GGCGGTGAAGCAGCTGGAC-3′, OTK2: 5′-GGGTGGAGACGTGGGTCTTGTC-3′) was synthesized

Table 1 Degenerated primers for STK type disease resistance gene analogs

Primer name	Type	Conserved amino acid sequences	Primer sequences
KF (upstream)	STK	FG (K V I S) VY (K R) G	TTYGGITCIGTITAYMRIGG
KR (downstream)	STK	D (V I) YS (F Y) G (V I M)	AYICCRWARCTRTAIAYRTC

Y = C T; R = A G; W = A T; M = A C; I = deoxyyinosine according to the most conservational region in OsSTK gene . For the amplification of OsSTK gene partial DNA sequences in different rice materials, the PCR conditions of 50 μl reaction mixture were modified and the same PCR procedure for all materials were used described as above in 1.2 except for the reanealing temperature is 60℃ . All the PCR products from different wild rice species were purified and directly sequenced by Sangon ltd . . Multiple DNA sequences analysis and alignment were made by the software DNASIS . Phylogenetic tree based on the OsSTK gene partial DNA sequences was constructed by the software CLASTALw1.83 .

1.4 Transcription level analysis of OsSTK gene

The young plant leaves of cultivated rice (Hexi 35), *O. rufipogon*, *O. officinalis*, *O. meyeriana*, *O. longistaminata* and *O. minuta* were used for the extraction of total RNA by a total RNA extraction kit (Gene Company) according to the manufacturer's protocol . The extracted total RNA was treated with RNase free DNase for 30 min at 37℃ to remove the genomic DNA . After 5 min denaturation at 70℃, the SuperScript first-strand synthesis system for RT-PCR kit (Clontech) was used to synthesize cDNA according to kit instructions . About 1 μg total RNA and Oligo dT that used to prime the cDNA were added to the synthesis reaction . One-tenth volume of the final cDNA (2 μl) was added to 20 μl PCR reaction mixture to amplify the OsSTK gene fragment as described above in 1.3 . The PCR products were detected by 1.0% agrose gel electrophoresis .

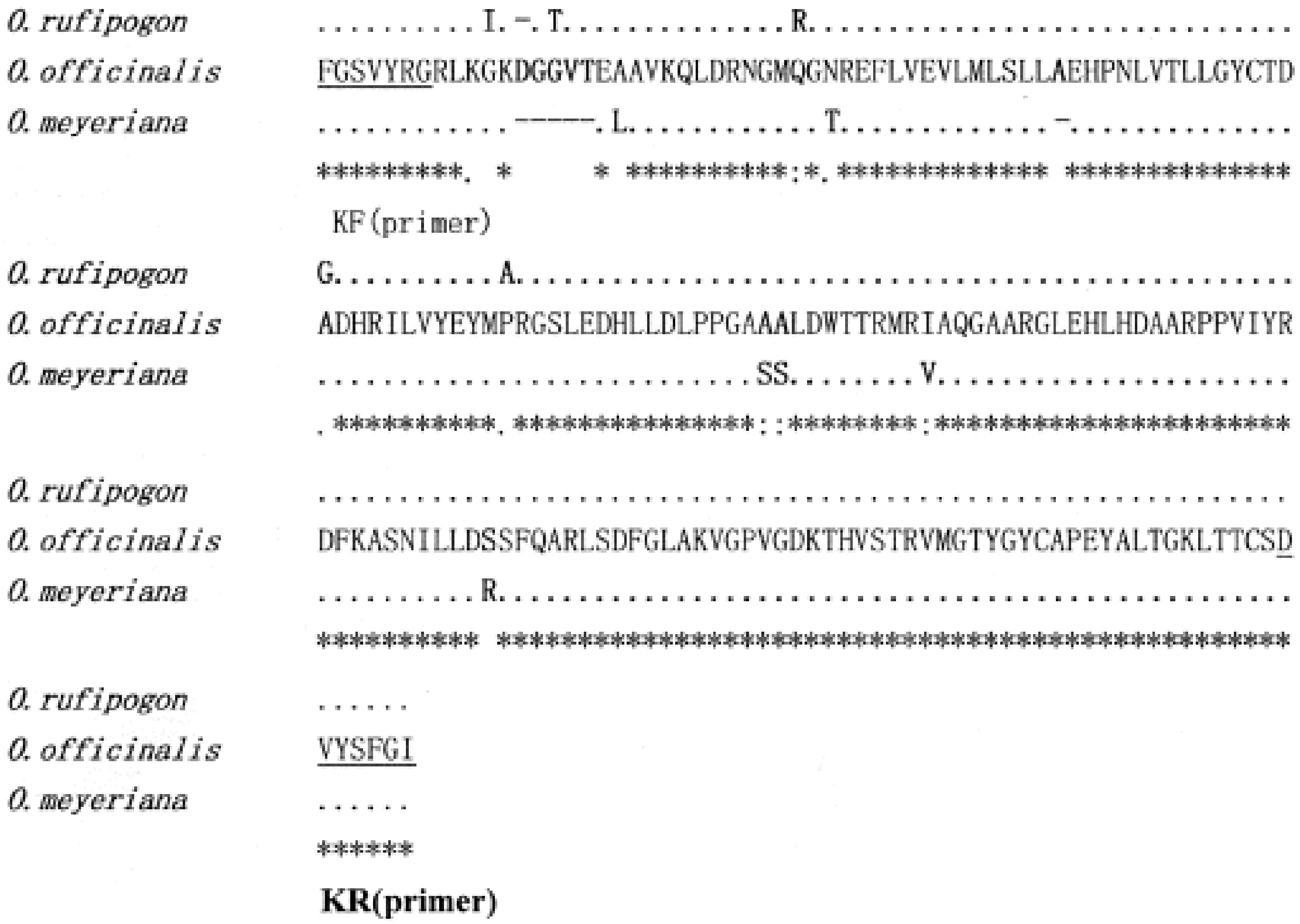
2 Results

2.1 Bait sequences from three species of wild rice

Many of the disease resistance analogues belonging to serine thronine protein kinase (STK) type have been cloned from different species of wild rice . Among these DNA sequences, 3 of them from wild rice *O. rufipogon*, *O. officinalis*, *O. meyeriana*, respectively, shared more than 91% identities of the deduced amino acid sequences (Fig.1) . These analogues may origin from one same gene or gene family (OsSTK) . There were 3 bp in *O. rufipogon*, and 18 bp in *O. meyeriana* deletion in these DNA sequences, respectively, compared to that of *O. officinalis* . But the deletion did not affect the predicted reading frame .

2.2 OsSTK gene in *O. sativa* (Japonica)

Using the cloned OsSTK DNA fragment from *O. rufipogon* as bait sequence, searching in the NCBI gene database with BLAST program, we found one cDNA clone (AK070226) without annotation and one BAC clone (gi: 53793517) from No . 9 chromosome of rice . All these clones contained DNA sequences that have more than 99% identities with the bait sequence,



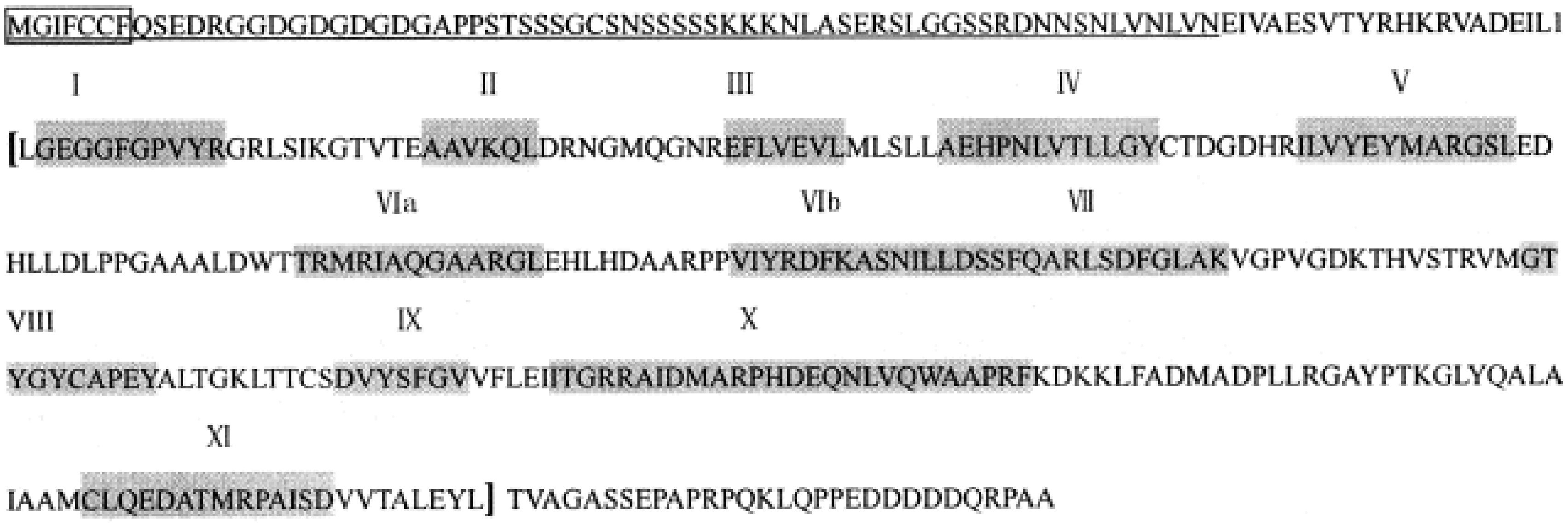
```
O. rufipogon      ..........I.-.T..............R..............................
O. officinalis    FGSVYRGRLKGKDGGVTEAAVKQLDRNGMQGNREFLVEVLMLSLLAEHPNLVTLLGYCTD
O. meyeriana      ............-----.L............T.............-..............
                  *********. *     * **********:*.************* **************
                   KF(primer)
O. rufipogon      G..........A................................................
O. officinalis    ADHRILVYEYMPRGSLEDHLLDLPPGAAALDWTTRMRIAQGAARGLEHLHDAARPPVIYR
O. meyeriana      ...........................SS........V......................
                  .**********.***************::********:**********************

O. rufipogon      ...........................................................
O. officinalis    DFKASNILLDSSFQARLSDFGLAKVGPVGDKTHVSTRVMGTYGYCAPEYALTGKLTTCSD
O. meyeriana      ..........R.................................................
                  ********** *************************************************

O. rufipogon      ......
O. officinalis    VYSFGI
O. meyeriana      ......
                  ******
                  KR(primer)
```

Fig . 1 Deduced amino acid sequences of three highly conserved STK type protein kinase analogues from three different species of wild rice
" * " indicates the amino acid identity and " . " shows the amino acid similarity . The underlined sequences are the positions of the degenerated primers

respectively. So, by analysis of these two clones we found the OsSTK gene consists of two exons, and the open reading frame separated by a short intron of 114 bp. OsSTK gene was predicted encoding a 419 amino acids cytoplasmic serine threonine protein kinase, with a predicted isoelectric point at 5.52. Like other protein kinase, the deduced OsSTK protein sequence contains all the invariant and highly conserved amino acids which characterize the 12 sub-domains (Fig. 2). The subdomain VIII of OsSTK was completely consensus to the classical serine threonine protein kinase. But the subdomain VIb and XI does not closely resemble either the consensus of typical serine threonine nor tyrosine protein kinase. The XI catalytic domain of OsSTK was much consensus to the tyrosine protein kinase (Table 2). The catalytic domain of OsSTK was flanked by N- and C-terminal regions of 114 and 30 residues, respectively. The N-terminal residues were abundant with serine and positively charged amino acids (lysine and arginine). Searching in protein databases using the program BLAST, we revealed no close relatives to the N-and C-terminal regions of OsSTK, but more than 150 characterized protein kinase sequences sharing identities greater than 50% with OsSTK catalytic domain were detected. Most of these protein kinases were not annotated yet. Among the annotated or partially annotated protein kinase, the most similar one was a auxin-regulated dual specificity cytosolic kinase from tomato (Lycopersicon esculentum) and a putative Avr9 Cf-9 rapidly elicited protein [*Oryza sativa* (japonica cultivar-group) XP_482765.1], analyzed by the software of Clustalw1.83.

2.3 Conservation analysis of OsSTK homologue gene in different species of wild rice

By using the specific primers (OSK1 OSK2), about 400 bp specific DNA sequences were amplified from all the Oryzae materials, including *O. sativa*, different types of *O. rufipogon*, *O. officinalis*, *O. longistaminata*, *O. minuta* and *O. meyeriana*. The OsSTK partial DNA sequences and corresponding deduced amino



MGIFCCFQSEDRGGDGDGDGDGAPPSTSSSGCSNSSSSSKKKNLASERSLGGSSRDNNSNLVNLVNEIVAESVTYRHKRVADEILI

I II III IV V

[LGEGGFGPVYRGRLSIKGTVTEAAVKQLDRNGMQGNREFLVEVLMLSLLAEHPNLVTLLGYCTDGDHRILVYEYMARGSLED

VIa VIb VII

HLLDLPPGAAALDWTTRMRIAQGAARGLEHLHDAARPPVIYRDFKASNILLDSSFQARLSDFGLAKVGPVGDKTHVSTRVMGT

VIII IX X

YGYCAPEYALTGKLTTCSDVYSFGVVFLEIITGRRAIDMARPHDEQNLVQWAAPRFKDKKLFADMADPLLRGAYPTKGLYQALA

XI

IAAMCLQEDATMRPAISDVVTALEYL] TVAGASSEPAPRPQKLQPPEDDDDDQRPAA

Fig. 2 Putative amino acid sequence of OsSTK gene in *O. sativa*

The framed N-terminal sequence showed the N-myristoylation motif; Roman numbers indicate the 12 corresponding catalytic sub-domains of the protein kinase. The serine, charged amino acids abundant N-terminal was under-lined. The GenBank accession number for the nucleotide sequence and the deduced amino acid sequence is DQ457011

Table 2 Comparison of the sub-domain **VI**b, **VIII** and **XI** of OSSTK, ADK1 with the corresponding sequences of typical serine thronine and Tyrosine types of protein kinases

Protein Kinase	VIb	VIII	XI
Serine threonine	D L K P E N	G (T S) XX (Y F) X AP E	XX (X) 6 RXXX
OSSTK	D F K A S N	G T Y G Y C A P E	CL (X) 6 RPAI
ADK1*	D I K P D N	G T A R Y A SV N	CR (X) 6 KPDY
AhSTKY	D L K S D N	G T Y R W MAPE	CW (X) 6RPPI
ATN1	D L K P E N	G T Y R W MAPE	CW (X) 6RPNF
Tyrosine	DL (R A) A (R A) N	XP (I V) (K R) W (T M) APE	CW (X) 6RPXF

acid sequences shares identities up to 91% and 90%, respectively. Phylogenetic tree based on the homologous of DNA sequences was constructed and displayed in Fig.3.

2.4 Transcription of OsSTK

About 400 bp specific DNA fragments can be amplified from all the Oryzae plants that we available by RT-PCR. For all the extracted tRNA were treated by RNase free DNase, the results proved the constitutively expression of OsSTK gene in all these plant leaves (Fig.4).

3 Discussion

PCR based technology together with bioinformatics was used to clone OsSTK coding for a novel protein kinase which may displays both serine thronine and tyrosine kinase characteristics. The properties of high conservation and constitutive expression of OsSTK gene in Oryzae plants imply the functional importance of OsSTK. The structure of OsSTK catalytic domain is similar to the first cloned disease resistant gene (R gene) PTO, which conferred to resistant *Pseudomonas syringae* pv. from tomato (Martin *et al.*, 1993). Pto gene encodes a 321 amino acids cytoplasmic serine thronine protein kinase and is proved to have the function of self-phosphorelation.

Dual specificity protein kinase which can phosphorylate serine, threonine and tyrosine was found in ADK1, ANK1 from Arabidopsis (Ali *et al.*, 1994; Tregear *et al.*, 1996), and in AhSTKY from peanut (Rudrabhatla and Rajasekharan, 2002). The sub-domains VIb, VIII and XI of OsSTK were closely resemble the ADK1, AhSTKY and ANK1 (Table 2). Sub-domain VIII of OsSTK contains 3 tyrosine residues. In animal tyrosine kinases, tyrosine residues between subdomains VII and VIII are thought to serve as sites for autophosphorylation (Hanks *et al.*, 1995).

Phylogenetic tree based on OsSTK gene partial DAN sequences was close to the taxation system of Oryzae plants (Fig.3). *O. sativa*, *O. rufipogon*, *O. longistaminata*

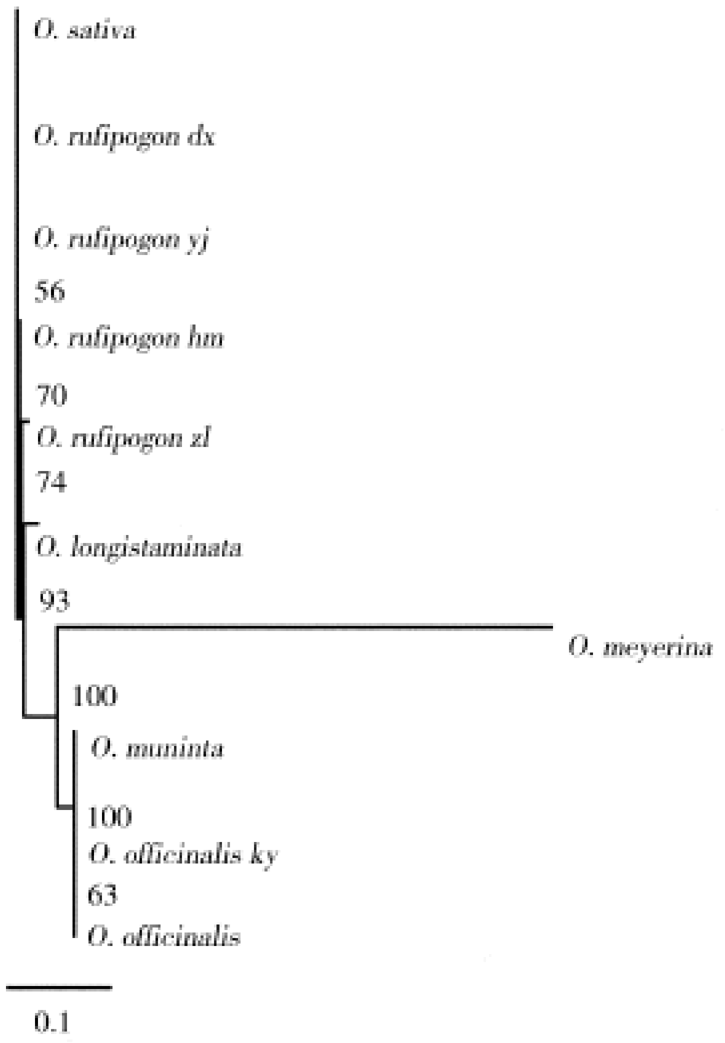


Fig. 3 Phylogenetic tree base on the partial sequences of OsSTK gene from different species and types of wild rice (Neighbor-Joint tree, constructed by the sofltware of CLASTAL w1.83, the numbers at every node were the support rate in 100 times of test)

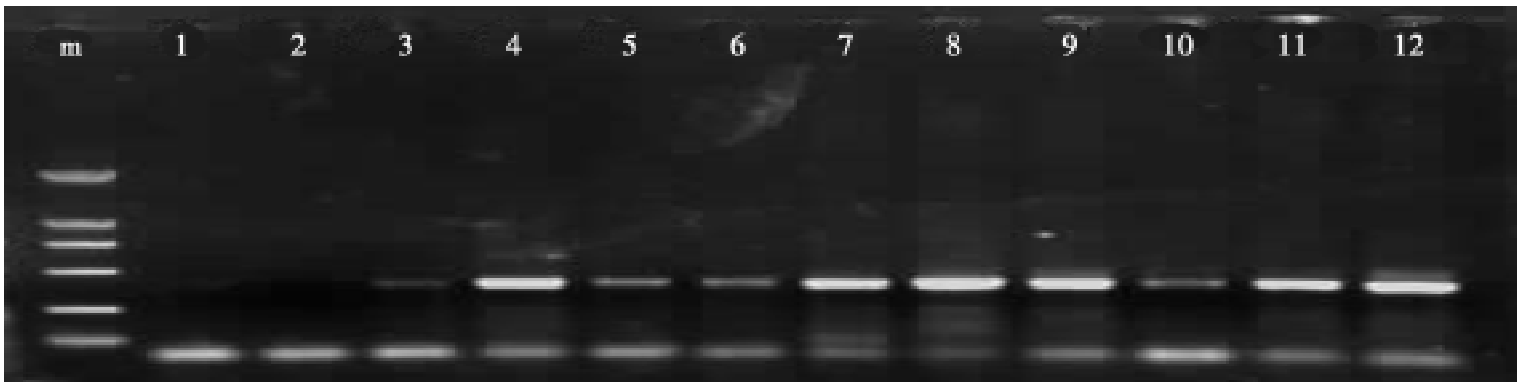


Fig. 4 RT-PCR results of OsSTK gene fragments from different species and types of wild rice and cultivated rice

m. indicates the DL2000 Markers; 1, 2. indicate the negative control and "1" is the negative control with no DNA plate, "2" is the negative control with RNase free DNase treated genomic DNA from *O. sativa* (hexi No. 35); 3. *O. saytiva* (hexi No. 35); 4. Dongxiang type of *O. rufipogon*; 5. *Oryza longistaminata* Chev er Roehr; 6. *Oryza minuta* J. s. presl; 7. *Oryza officinalis* Wall; 8. *Oryza meyeriana* Ball; 9. *Oryza officinalis* Wall; 10. Yuanjiang type of *O. rufipogon* Griff; 11. Erect type of *O. rufipogon* Griff; 12. Red awn type of *O. rufipogon* Griff belonging to the same AA genotype (Vaughan *et al.*, 2003), were located in the nearest position in the tree. *O. meyeriana* belonging to GG genotype that located in relatively farther position from other species of Oryzae plants. *O. officinalis* and *O. munita* have partially same genotype, and located in a relative close position in the tree. Becauses they belong to the CC and BBCC genetypes, respectively.

Besides a specific peptide at the N-terminal, moreover, OsSTK contains an MGIFCCF motif which matches the (MGXX [S T A G C N] X) consensus for N-myristoylation (Towler and Gordon, 1988). This co-translational modification is catalized by a myristoy-CoA: protein N-myristoyl transferase (NMT), for which an activity was found in wheat (Heuckeroth *et al.*, 1988). The previously studied animal, yeast and wheat enzymes were found to myristoylate the amino group of the glycine at position +2, the sequence context greatly affect the efficiency of reaction. It was found in particular that the presence of either a serine, a cysteine or a second glycine at position +6, the absence of a praline or a charged amino acid at position +3 and the absence of praline at position +7 were important (Casey, 1995). And these characters were found in OsSTK. N-myristoylation often has an important functional role, either for protein-protein interaction or for protein-membrane attachment (Ishitani *et al.*, 2000). N-myritoylation motif was recently found in calcineurin B-like proteins (CBLs) which acted in CBL CIPK signaling system to mediate a variety of responses to environmental stimuli in plants (Gao *et al.*, 2008). It is therefore possible that this co-translational modification might permit membrane association in the case of OsSTK. The specific N-terminal sequences, abundant with serine, basic and charged amino acids, may have specific role in cell positional and protein recorgnizition. No T [D E] Y module was found between sub-domain VII and VIII in OsSTK, which is necessary for activation of MAP kinase, an evidence that powerfully supported their reliability as the members of the rice MAPK family (Liu *et al.*, 2007). Therefore, OsSTK doesn't belong to the MAPK family. The real function of OsSTK gene is deserved to be further confirmed.

References:

Ali N, Halfer U, Chua NH, 1994. Cloning and biochemical characterization of plant protein kinase that phosphorylate serine, threonine, and tyrosine [J]. *The Journal of Biological Chemistry*, 269: 31626—31629

Casey PJ, 1995. Protein lipidation in cell signaling [J]. *Science*, 268: 221—225

Doyle JJ, Doyle JL, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue [J]. *Phyto Chemistry*, 19: 11—15

Gao P, Zhao PM, Wang J *et al.*, 2008. Co-expression and preferential interaction between two calcineurin B-like proteins and a CBL-interacting protein kinase from cotton [J]. *Plant Physiology & Biochemistry*, 46: 935—940

Hanks SK, Quinn AM, 1991. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members [J]. *Methods Enzymology*, 200: 38—61

Hanks SK, Hunter T, 1995. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification [J]. *FASEB J*, 9: 576—596

Heuckeroth RO, Towler DA, Adams SP *et al.*, 1988. 1-(ethylthio) undecanoic acid, a myristic acidanalogue of altered hydrophobicity which is functional for peptide N-myristoylation with wheat germ and yeast acyltransferase [J]. *The Journal of Biological Chemistry*, 263: 2127—2133

Ishitani M, Liu J, Halfter U *et al.*, 2000. SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding [J]. *Plant Cell*, 12: 1667—1678

Julie M, John S, Walker C, 1995. Plant protein kinase families and signal transduction [J]. *Plant Physiology*, 108: 451—457

Liu QP, Xue QZ, 2007. Computational identification and phylogenetic analysis of the MAPK gene family in *Oryza sativa* [J]. *Plant Physiology & Biochemistry*, 45: 6—14

Martin GB, Brommonschenkel SH, Chunwongse J, 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato [J]. *Science*, 262: 1432—1436

Natalia GE, Chou CF, Lin WW *et al.*, 1998. The G11 gene located in the major histocompatibility complex encodes a novel nuclear serine threonine proteinkinase [J]. *The Journal of Biological Chemistry*, 273: 30954—30960

Rudrabhatla P, Rajasekharan R, 2002. Developmentally Regulated Dual-Specificity Kinase from Peanut That Is Induced by Abiotic Stresses [J]. *Plant Physiology*, 130: 380—390

Towler DA, Gordon JI, 1988. The biology and enxymology of eukaryotic protein acylation [J]. *Annual Review of Biochemistry*, 57: 69—99

Tregear JW, Jouannic S, Schwebel-Dugue N *et al.*, 1996. Kreis M. An unusual protein kinase displaying characteristics of both the serine threonine kinase and tyrosine families is encoded by the *Arabidopsis thaliana* gene ATN1 [J]. *Plant Science*, 117: 107—119

Vaughan DA, Morishima H, Kadowaki K, 2003. Diversity in the *Oryza genus* [J]. *Current Opinion of Plant Biology*, 6: 139—146